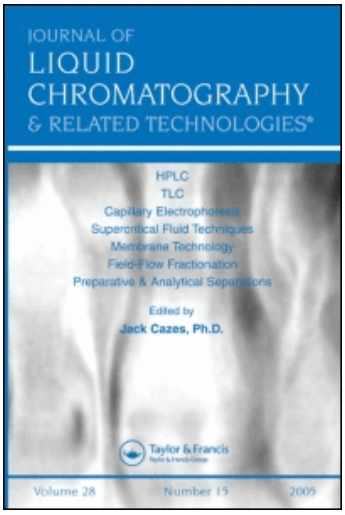

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Supercritical Fluid Extraction of Mycotoxins from Feeds with Analysis by Lc/Uv and Lc/Ms

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SUPERCRITICAL FLUID EXTRACTION OF MYCOTOXINS FROM FEEDS WITH ANALYSIS BY LC/UV AND LC/MS

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ABSTRACT

The use of supercritical fluid extraction with supercritical carbon dioxide and a matrix modifier is described for the isolation of three trichothecene mycotoxins from yellow corn meal and rolled oats. The quantity of supercritical carbon dioxide used in each extraction was 30 mL which contained 5% methanol used as a modifier. The pressure was maintained at 550 atm., and the temperature of the extraction chamber was set at 60 degrees. Recovery of the target analytes from cereal and corn feed by supercritical fluid extraction (SFE) varied from 85 to 95 % depending on the target compound. The mycotoxins, deoxynivalenol (DON), deacetoxyscirpenol (DAS) and T-2 toxin (T-2) were monitored in the SFE extracts by high performance liquid chromatography with UV-detection (HPLC-UV) or ion spray mass spectrometry under full-scan, selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes. There are significant restrictions in the HPLC-UV detection limits for T-2 and DAS, in particular, due to their low UV absorbance. With

HPLC-UV and also HPLC-MS in the full-scan mode of acquisition the considerable interference from co-eluting fat-soluble compounds precluded detection of these compounds in feed extracts. In the SIM and SRM LC/MS modes the target analytes could be readily detected at the 250 ppb level in the cereal SFE matrix extracts.

INTRODUCTION

Trichothecenes are sesquiterpenoid mycotoxins produced by a variety of species of *fusarium* fungi. These fungi have been established as plant-pathogenic fungi which invade various agricultural crops and plants. These fungi thus have the potential for producing tricothecene mycotoxins in grains and cereals. Trichothecenes show a wide range of toxicity, which is dependent on the structure of the molecule. Over 150 trichothecenes have been isolated and characterized, but it is still a challenging analytical task to isolate and characterize these compounds from foods and feeds.[1,2]

Conventional methods for isolation of trichothecenes involve extensive and time-consuming sample preparation steps. According to a recent survey, two thirds of the analysis time is devoted to sample preparation and this step accounts for at least one-third of the errors generated during the performance of an analytical method.[3] Supercritical fluid extraction (SFE) has shown great potential in offering shorter extraction times with good recoveries and low consumption of organic solvents. Supercritical carbon dioxide (SC-CO_2) is the most widely used fluid for supercritical techniques because it is non toxic, nonflammable and inexpensive. Supercritical carbon dioxide provides extraction capability at relatively low temperatures and pressures because its critical pressure (7.38 MPa) and temperature (31.1°C) are relatively low.[4] This enables the use of low extraction temperatures thereby reducing the risk of analyte degradation during extraction. Due to favorable physical properties the supercritical fluids are also amenable with other chromatographic techniques.[5]

Supercritical fluid extraction has been used for industrial-scale separation and isolation of a variety of compounds.[6] An increasing number of quantitative applications of SFE especially for the extraction of environmental pollutants has been reported in recent years.[7-10] SFE is also utilized in the field of food science to isolate not only natural food components including fat,[11] cholesterol,[12] and volatiles,[13] but also unnatural components like organic contaminants including pesticides and PAHs .[14,15]

Mycotoxins are often separated, identified and quantitated using thin layer chromatography,[16] thin layer chromatography mass spectrometry,[17] gas chromatography-mass spectrometry,[18-22] high performance liquid chromatography (HPLC) methods with fluorescence[23,24] or light scattering[25] detection. Conventional liquid and gas chromatography methods, however, suffer serious drawbacks. The sensitivity of HPLC is limited since most trichothecenes have minimal fluorescent or ultraviolet absorbing properties. In the case of GC-methods, derivatization is often required, which may cause problems with quantitative analysis procedures. HPLC combined with mass spectrometry via thermospray, plasmaspray or fast atom bombardment[26-29] have also been reported.

Only limited studies have been published with the use of supercritical fluid extraction for the isolation of mycotoxins or with a comparison of different identification methods. Supercritical fluid chromatography (SFC) combined with UV-detection or mass spectrometry via the moving belt interface has been used for the determination of some *Fusarium*-toxins.[30] Kalinoski *et al.*[31] have used SFE combined with direct fluid injection mass spectrometry (DFI/MS) for the determination of trichothecenes from wheat samples. SFE is also used in the isolation of aflatoxin B_1 from field-inoculated corn[32] and peanut meal.[33] In both cases analytes were monitored using HPLC equipped with a fluorescence detector.

The aim of this study was to evaluate the feasibility of supercritical fluid extraction in the isolation of some trichothecene toxins from cereals and feed. A comparison of HPLC-UV, HPLC/MS and HPLC/MS/MS was made to determine the preferred method for monitoring the analytes isolated by SFE. Extracts were analyzed without further purification. One of the topics of this report is to describe how and why HPLC/APIMS/MS may be a preferred means of developing routine, high-sensitive methods for analyzing biological samples using limited sample preparation procedures.

MATERIALS AND METHODS

Samples and Standards

Samples, rolled oats and yellow cornmeal, were purchased from a local supplier (Ithaca NY, USA). The feed samples were available in the laboratory from previous veterinary toxicology case studies. All samples were ground to a fine powder with a homogenizer (Waring commercial blender, Model 7011S, Fisher Scientific, Rochester, NY). These samples were stored in the capped

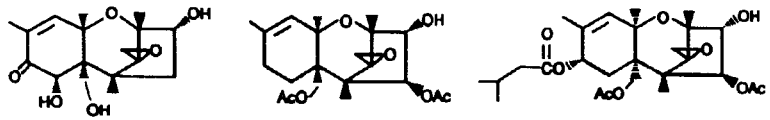

Figure 1. Structures of three trichothecenes investigated.

Corning 250 mL, 8oz polypropylene containers, (Fisher Scientific, Rochester, NY) at the room temperature. A standard stock solution of the three mycotoxins (500 ng/μL methanol) was prepared from pure standards purchased from Sigma Chemical Co (St. Louis, MO, USA). The stock solution was diluted 1:100 with methanol. All reagent-grade solvents, buffers and common reagents were purchased from Fisher Scientific (Rochester, NY, USA). The mycotoxins used in this study were deoxynivalenol (DON), deacetoxyscirpenol (DAS) and T-2 toxin (T-2). The structures of these compounds are shown in Figure 1. Fortification of the samples was done by injecting an appropriate amount of the diluted standard stock solution of mycotoxins into the sample in the extraction vessel. The concentrations prepared were 250, 500 and 1500-ppb. Repeatability of SFE was based on the data obtained from the tests, where samples were spiked before and after supercritical fluid extraction. The LC/MS ion current profiles for the extract of the fortified sample and the post-extract spike of a control sample were compared to calculate the repeatability.

Supercritical Fluid Extraction

Four grams of sample were extracted with an ISCO model 100 DX dual syringe pump system coupled with an ISCO SFX 2-10 extractor and a two-channel adjustable restrictor device (ISCO Inc., Lincoln, NE, USA). A layer of anhydrous sodium sulfate was placed in a 10mL extraction vessel followed by four grams of sample, and the remaining void filled with additional anhydrous sodium sulfate. SFC-grade CO_2 was used from an aluminum cylinder charged with a helium head pressure and equipped with a dip tube (Scott Specialty Gases, Plumbsteadville, PA, USA). The ISCO SFE pump head was maintained at +5°C with an external cryostat (Neslab, Model RTE-110). The other pump of the ISCO system was used for adding methanol as a modifier. Extraction conditions used were as follows: pressure 550 atm (1 atm = 0.10132 MPa), extraction temperature was 60°C, restrictor temperature was 65°C, fluid volume used was 30 mL with 5 % of HPLC-grade methanol. The flow rate of

supercritical fluid was maintained at 1.2 mL/min. The syringe pump that delivered the CO_2 was refilled after each extraction. The analytes were collected by bubbling the SFE-extracted material into 10 mL of methanol. Extracts were defatted with 5 mL of hexane, which was removed by aspiration with Pasteur pipette. Samples were evaporated to dryness under nitrogen, diluted with 500 μL of mobile phase and stored at + 5°C prior HPLC-UV and HPLC/MS analysis.

High Performance Liquid Chromatography

Reconstituted SFE extracts as well as standard mixtures were injected into a Waters HPLC system, Model # 600-MS (Waters Inc., Milford, MA, USA) using a Rheodyne 7125 injector (Rheodyne Inc., Cotati, CA, USA) equipped with 5 μL sample loop. The mobile phase was methanol: acetonitrile:ammonium acetate (3 mM) 45:5:50 (v/v/v). The HPLC flow rate was 200 μL/min. and the column utilized was 2 mm ID x 10 cm, 5 μm, 100 Å, BETASIL C18 (Keystone Scientific Inc., Bellefonte, PA, USA). A variable wavelength detector, Spectroflow (ABI Analytical Kratos Division, Ramsey, NJ, USA) was used for monitoring the three mycotoxins, deoxynivalenol (DON), diacetoxyscirpenol (DAS) and T-2 toxin (T-2) at 220 nm.

Mass Spectrometry

A PE Sciex API 300 LC/MS/MS System (Perkin Elmer Sciex Instruments, Thornhill, Ontario, Canada) equipped with an ion spray LC/MS interface was used for all mass-spectrometry studies. The HPLC flow was maintained at 200 μL/min. without post-column splitting of the HPLC effluent. The ammonium adduct ions of the three mycotoxins were detected by HPLC/MS under full-scan and selected ion monitoring (SIM) modes. When HPLC/MS/MS experiments were undertaken, the mass spectrometer was operated in the product ion mode under selected reaction monitoring (SRM) conditions using the ammoniated molecules as the precursor ions. The mass range in full-scan experiments was 50-600 Da using a step size of 0.2 Da. Under scanning conditions a scan rate of 4 s per scan was used, while under SIM conditions a dwell time of 200.0 msec was used. Two product ions from each compound were monitored under SRM conditions. For all MS/MS experiments nitrogen was used as the collision gas at a setting of 3 on the PE-SCIEX API 300. Quantitation was performed using reference to an external standard analyzed under identical experiment conditions.

RESULTS AND DISCUSSION

Supercritical fluid extraction with pure CO_2 is a useful approach for extraction of relatively nonpolar compounds. However, when polar compounds *e.g.* trichothecenes are present in a sample, either ultrahigh pressures (> 10 000 psi) or organic modifiers must be used. In this work very high pressures were not available, so the highest pressure available from the ISCO system was used combined with MeOH as a modifier for the supercritical carbon dioxide. Under these conditions trichothecenes are readily extracted from grain samples by supercritical fluid extraction. However, the aggressive conditions used also recovers many other interfering compounds, which leads to considerable chemical interference when UV detection is used following HPLC separation. Furthermore, the detection of trichothecenes by HPLC-UV is relatively nonspecific due to the lack of strong chromophores. These problems could potentially be resolved by using further purification and derivatization procedures or by employing more specific detection such as mass spectrometry.

The SFE conditions used in this work were as follows: pressure 550 atm (=8085 psi), extraction temperature 60°C and restrictor temperature 65°C. Thirty mL of supercritical CO_2 doped with 5 % methanol were used for each sample extraction. The optimum conditions used were based on our systematic studies designed to determine the optimum pressure and temperature to afford the best recovery of the target compounds with a minimum of chemical interference. Higher pressures and temperatures as well as varying quantities of the MeOH modifier were used, but coeluting lipophilic compounds interfered with UV-detection. As can be seen from Figure 1, DON is perhaps the most polar mycotoxin while DAS and T-2 are less polar. T-2 and DAS do not, however, have functional groups which absorb or fluoresce at 220 nm, whereas DON has somewhat better UV absorbance. Due to the relatively weak absorbance of these three compounds, interfering chemical constituents in the extract can easily interfere with the detection of the target analytes in the supercritical extracts. Recoveries of SFE were calculated by spiking samples before and after SFE. Spiking levels in all cases were 250, 500 and 1500-ppb.

Recovery studies with references to external standards showed that DON gave the highest recovery (95 %) while DAS and T-2 each were recovered at the 85 % level under the SFE conditions used. The results were a little surprising given that less polar compounds are usually isolated more easily with SFE than polar compounds. When multiple samples were extracted by SFE, the variable restrictor was wiped clean with MeOH to ensure minimal carryover between samples. Systematic studies with blank samples showed no evidence for carryover between samples using either HPLC/UV of HPLC/MS.

Table 1

Mass Spectral Data of Three Trichothecenes Investigated

Compound	$[M + H]^{\pm}$	$[M + NH_4]^{\pm}$	Product Ions
	m/z	m/z	m/z
DON	297	314.2	249.0 and 297.0
DAS	367	384.1	247.0 and 307.0
T-2	467	484.1	245.0 and 305.0

HPLC conditions were based on our earlier studies[34] with the exception that ammonium acetate was used in this work to afford ammonium adduct ions from the ion spray LC/MS experiments. In this study isocratic elution of the test compounds was used. The HPLC conditions were chosen so that the elution of three toxins investigated could be accomplished in a minimum time period. The organic solvent content in the mobile phase was also maintained as high as possible to afford maximum sensitivity for electrospray ionization. The total HPLC analysis time was 6 min. per sample. The elution order of the analytes was as follows: DON, DAS and T-2. First, UV-detection was used at 220 nm, but with this wavelength DAS and T-2 could not be detected in a 500 ppb sample extract. The chemical background in the HPLC chromatogram was sufficiently high that even DON cold not be detected when the SFE extracts from real samples were analyzed. A preferred approach is to use a more specific detection system such as mass spectrometry.

Ion spray mass spectrometric analysis was performed in three different acquisition modes. First, full-scan acquisition was carried over the mass range of 50-600 amu Da. To gain higher specificity as well as sensitivity the instrument was then operated in the selected ion monitoring mode (SIM). The ions selected were the ammonium adduct ions of three trichothecenes. These ions as well as the corresponding protonated molecules are listed in Table 1. The ammoniated adduct ions were selected because of their high abundance in the mass spectra of these compounds under the experimental conditions used. Finally, to obtain a combination of high sensitivity as well as high specificity the selected reaction monitoring (SRM) mode of operation was used. In these experiments nitrogen was used as a collision gas in the central collision cell positioned between the two quadrupole mass analyzers. When the precursor ions derived from electrospray ionization are focused into the collision cell, fragmentation occurs that affords a high level of specificity for the target compounds. In addition, the pre selection of the ammoniated adduct ions from

each target compound affords a significant reduction of chemical noise thereby providing a higher signal-to-noise in the final ion current profile of the chromatogram. A total of six ions were monitored in these experiments since two unique product ions for each of the three target compounds were monitored. The relevant product ions are listed in Table 1.

Figure 2A-D shows the results obtained from the four HPLC analyses described above. In each case, supercritical fluid extraction was applied for isolation of the three trichotecenes DON, DAS, T-2 from cereal samples containing 500-ppb levels of each compound. Figure 2A shows the HPLC chromatogram when UV-detection was used. The sample used was rolled oats spiked with 500-ppb of each mycotoxin. The total run time was 20 min. DON could be observed at the 500 ppb level only as a shoulder on the first peak. DAS and T-2 were not detected in the HPLC/UV chromatogram under these experimental conditions. Neither quantitative nor qualitative determination of these compounds could be possible with this experimental approach.

At the 250-ppb level DON was not detected at all by HPLC/UV (data not shown). A more specific detector such as mass spectrometry can provide a more reliable way to detect compounds present in complex mixtures. Figure 2B-D shows an example of this point. These data were obtained by the LC/MS analysis of an SFE extract of rolled oats spiked with 250-ppb of each compound. Figure 2B shows the chromatogram obtained from the full-scan LC/MS analysis of the supercritical fluid extract of ground oats. Although each of the target analytes may be detected in this LC/MS TIC chromatogram, there is still considerable chemical interference. In spite of this, one may obtain background-subtracted mass spectra from each of the target compounds in this chromatogram (data not shown) which readily verify the molecular weight for each compound.

Figure 2 (right). HPLC/UV and HPLC/MS chromatograms obtained from the determination of the target trichothecenes isolated from rolled oats fortified at the 500 ppb level (HPLC/UV) and 250 ppb levels (HPLC/MS) of the three test compounds.
A) HPLC/UV chromatogram obtained from the analysis of a supercritical fluid extract of rolled oats spiked with 500 ppb levels of the three test compounds. B) Full-scan LC/MS TIC chromatogram obtained from the analysis of a supercritical fluid extract of rolled oats spiked with 250 ppb levels of the three test compounds. C) SRM LC/MS chromatogram obtained from the analysis of a supercritical fluid extract of rolled oats spiked with 250 ppb levels of the three test compounds. See text for experimental details.

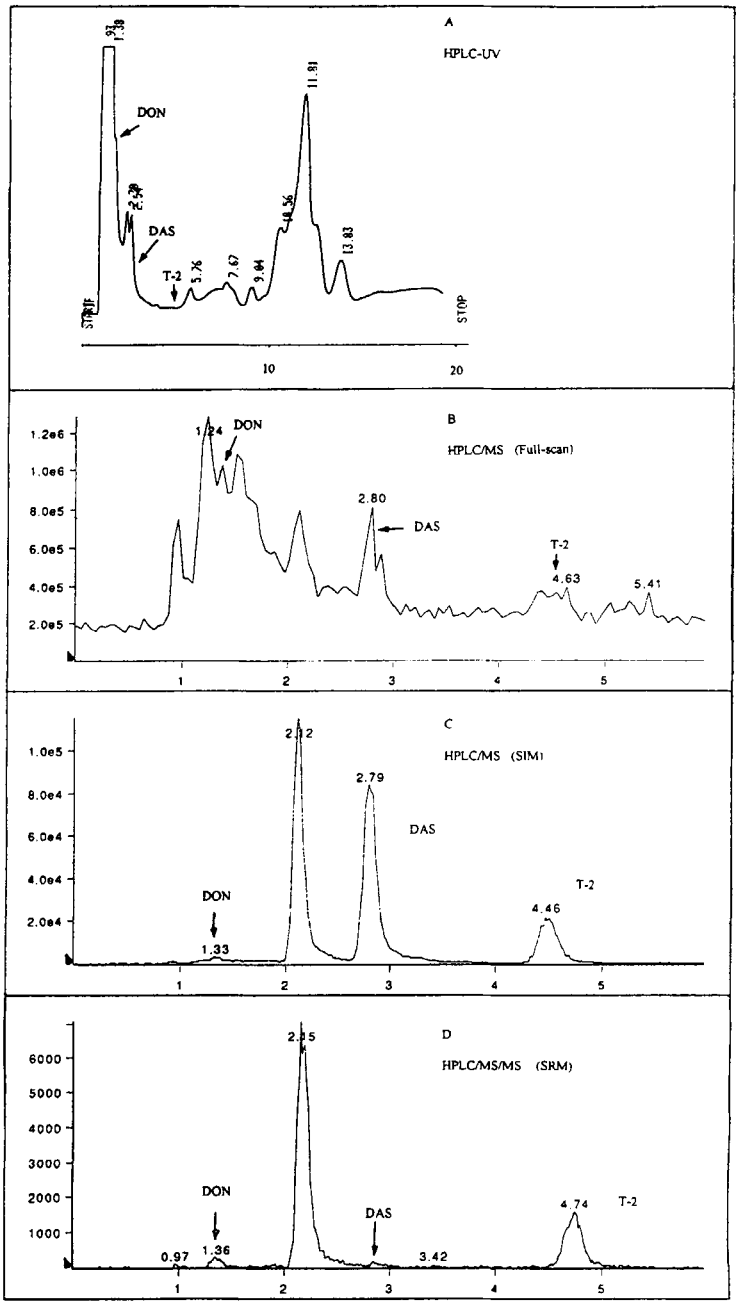

Figure 2C shows a much improved ion current chromatogram signal-to-noise ratio when SIM LC/MS analysis of the same sample is performed. The large peak eluting at 2.12 min. is an endogenous compound that is detected under these conditions since it appears to have ions common to the target compounds. Finally, the results from the SRM LC/MS analysis of the same sample are shown in Figure 2D. The signal-to-noise ratio improves further in this experiment, although the gains from tandem mass spectrometry are not as large as often observed. However, since two precursor-product ion transitions are monitored for each compound (Table 1), one has considerably more specificity from these data than from the SIM results.

Unfortunately, the response from DON under these positive ion spray LC/MS conditions is not as good as for the other compounds. This is sometimes observed when compounds have significantly different proton or ammonium ion affinities. In addition, the high specificity of MS/MS has not removed the large component eluting at 2.12 min. However, the ion current profiles shown in Figure 2C,D display a significantly improved sensitivity and specificity over the LC/UV chromatogram shown in Figure 2A. Similar results were obtained when the sample extracted was yellow cornmeal spiked with 250-, 500- and 1500-ppb of each mycotoxin using these same experimental conditions. In each case only the SIM- or SRM LC/MS analyses reliably detected the target analytes.

It is clear from the above data that mass spectrometry provides a more selective and sensitive method for analyzing samples containing these trichothecenes. The total ion current chromatogram (Figure 2B), however, shows only slightly more information than the HPLC/UV chromatogram shown in Figure 2A. However, when either SIM or SRM LC/MS are used, all three mycotoxins could be easily detected and identified at the 250 ppb level (Figure 2C and D). Good sensitivity and specificity were achieved in the SIM LC/MS determination of trichothecenes in the SF-extracts (Figure 2C), but the lack of fragmentation decreases the reliability of the identification and does not provide any fragmentation information that can facilitate qualitative confirmation of these compounds. It is for these reasons that more selectivity is needed. Figure 2D shows the most powerful feature of MS if tandem mass spectrometry (MS/MS) is available. The ion spray LC/MS/MS experiment was set to monitor the precursor-product ion transitions characteristic of the three target compounds. This experiment, therefore, provides very high specificity for the qualitative determination for each of the three compounds even when they are present in complex matrix. It should also be true that improved detection limits could be achieved for quantitative analyses using this approach, provided an appropriate internal standard was used.

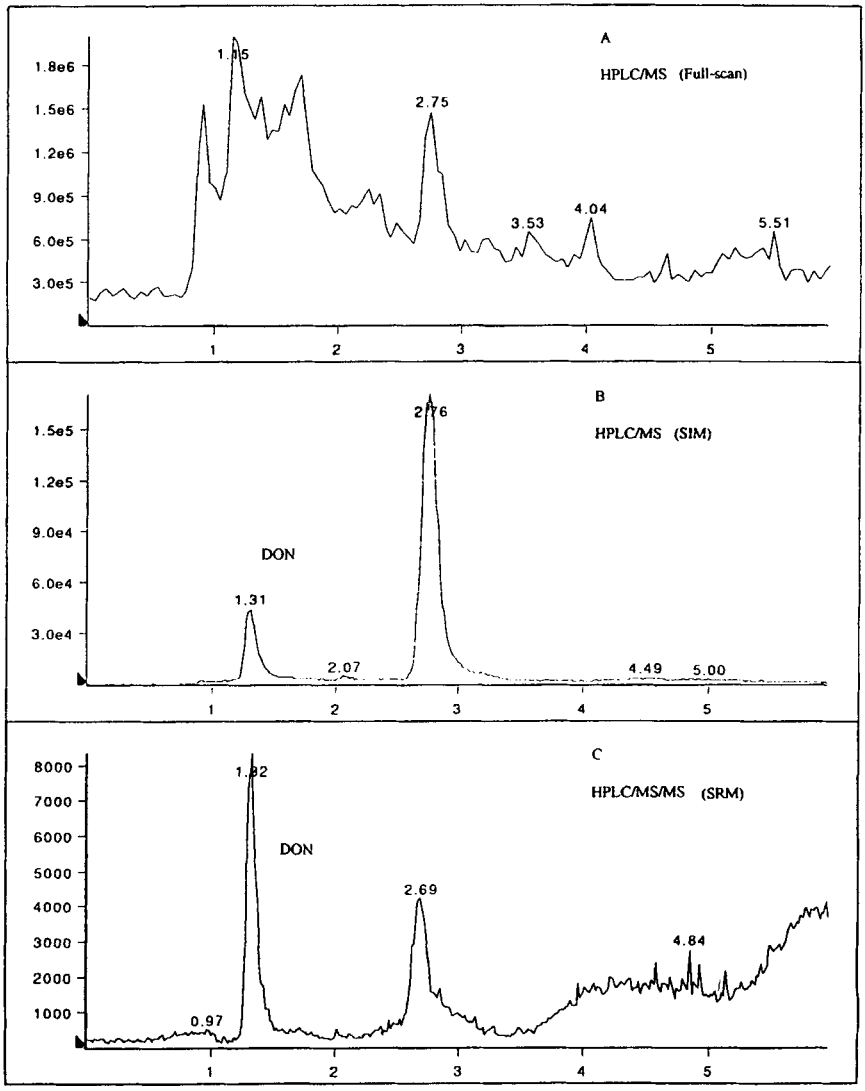

Figure 3. HPLC/MS analysis of corn feed spiked with 1 ppm levels of the three test compounds. A) Full-scan LC/MS TIC from the analysis of the supercritical fluid extract of the sample. B) SIM LC/MS analysis of the supercritical fluid extract of the sample. C) SRM LC/MS analysis of the supercritical fluid extract of the sample. See text for experimental details

Figure 3 shows the LC/MS and LC/MS/MS chromatograms obtained from a supercritical fluid extract of a feed sample fortified at the 1 ppm level. The SFE extract was yellow in color and a few droplets of fat were observed in the collection tube. This material was removed by extraction with hexane before LC/MS/MS analysis of the SFE extract. The DON in this corn feed is easily determined using this approach. Figure 3A shows the corresponding full-scan TIC chromatogram and Figure 3 B and C show the SIM and SRM LC/MS chromatograms, respectively. HPLC/UV analysis was not attempted due to the high levels of interfering endogenous chemical background present in the supercritical fluid extract.

CONCLUSIONS

The results described show that trichothecenes may be extracted from cereal and grain samples by supercritical fluid extraction with high recoveries. If the analytes are monitored by high performance liquid chromatography-tandem quadrupole mass spectrometry via atmospheric pressure ionization interface, the target compounds may then be quantitatively and qualitatively determined with an LC/MS sample throughput of approximately 10 samples per hour. The limit of detection with HPLC-UV was 500-ppb for DON and not detectable for T-2 and DAX. The detection limit using HPLC/MS/MS techniques are estimated to be in the low ppb range for each of the three analytes. HPLC-UV analyses of the extracts were hampered by co-eluting fat-soluble compounds that produced high chemical interference with the target compounds. LC/MS and LC/MS/MS techniques circumvent this problem by providing much higher specificity for the test compounds in the presence of high chemical interference.

SFE-API/HPLC/MS/MS analyses are an efficient and rapid means of analyzing biological samples for the determination of unknown and target compounds. The API source operates at ambient temperature and easily handles HPLC column flow rates of 200 μL/min. without post-column splitting of the HPLC effluent. Results presented in this work suggest that the sensitivity and ruggedness of LC/MS/MS is suitable if not preferred when combined with the merits of supercritical fluid extraction of feed and cereal matrices containing the target compounds studied in this work.

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